PHYTOCHEMICAL ASPECTS OF FERN SYSTEMATICS

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Abstract

The use of chemical data in fern systematics follows three main approaches: physiological studies, protein studies, and comparisons of secondary metabolite distributions. Physiological studies are useful at generic and higher taxonomic levels. These investigations include work on: (1) fern antheridogens and the differential response of various fern families to antheridial induction by these hormones, (2) the phenol glucosylation pathway, in which vascular plants possess the ability to glucosylate exogenously administered phenolic compounds, while non-vascular plants do not, and (3) differences in *D*-methionine metabolism in vascular and non-vascular plants. Protein work in ferns is limited and consists mainly of serological and electrophoretic studies. Secondary metabolites remain the largest body of chemical data available for chemosystematic research and are most effectively used at generic and lower taxonomic levels. Studies of acylphloroglucinol compounds in *Dryopteris* and some recent work with flavonoids in the fern genera, *Gymnopteris* and *Hemionitis*, are described as examples of chemosystematic studies employing secondary metabolites.

The development of new analytical techniques, such as paper and gas chromatography, and electrophoresis, has done much to expand the use of phytochemical data in plant studies (Alston, 1969; Alston & Turner, 1963). While this type of chemical information has been employed extensively in gymnosperm and angiosperm systematics, its use in fern systematics is still relatively recent. Yet, even a cursory look at the types of chemical constituents produced by ferns clearly shows great potential for such studies (Berti & Bottari, 1968). From the literature available, chemosystematic research on ferns appears to follow three major approaches:

(1) Physiological studies: examination of the effects on physiological and biosynthetic pathways in ferns and their allies, upon addition of natural or synthetic substances to the growing plant.

(2) Protein studies: systematic comparisons of proteins in ferns using various serological and/or electrophoretic techniques.

(3) Secondary metabolites: comparison of compounds which accumulate in the tissues of ferns and apparently are not involved in primary metabolic pathways, but differ qualitatively and/or quantitatively enough in structural variation as to be useful in systematic studies.

Within the limits of this discussion we will endeavor to examine several examples of each of these three approaches.

Physiological Studies

One of the more significant physiological works is that by Voeller on sex hormones in ferns (Voeller, 1971; Voeller & Weinberg, 1969; Weinberg & Voeller, 1969). Working with *Pteridium aquilinum*, Voeller succeeded in isolating and purifying a sex hormone, which was subsequently named Antheridogen-A. As described by Voeller (1971) and other earlier workers, some of the fern gameto-

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TABLE 1. Antheridial and germination responses to fern hormones.[a]

	Antheridial formation			Germination responses				
	Antheridogen		GA_3[b]	Light	Dark	Dark/Antheridogen		Dark/GA_3[b]
Family	A	B				A	B	
1. Aspidiaceae	+	–	–	+	(+)	+	–	–
2. Pteridaceae	+	–	–	+	(+)	+	–	–
3. Adiantaceae	+	–	–	+	–	+	–	–
4. Blechnaceae	+	–	–	+	–	+	–	–
5. Davalliaceae	+	–	–	+	–	+	–	–
6. Polypodiaceae	(+)[c]	–	–	+	(+)	(+)	–	–
7. Cyatheaceae	–	–	–	+	–	–	–	–
8. Osmundaceae	–	–	–	+	–	–	–	–
9. Schizaeaceae	–	+	+	+	–	–	+	+

[a] This table is a summarization of data taken from Voeller's papers.

[b] GA_3—gibberellic acid.

[c] Indicates that only one species gave a positive reaction in an otherwise negatively reacting family.

phytes under axenic culture grow faster than others and produce this hormone which in turn induces antheridial formation in the remaining, slower-growing gametophytes. Antheridogen-A was also found to induce production of an intragametophytic inhibitor in the larger hormone-producing gametophytes. This inhibitor stops antheridial formation in these large gametophytes which then become archegoniate. A second hormone, Antheridogen-B, was also found, and its physiological affects as well as spore germination responses to light, darkness, and gibberellic acid were also examined.

In his review of this work, Voeller (1971) clearly emphasizes the potential for physiological and morphogenetic studies of plant growth using ferns, especially since the distinct gametophytic stage can be grown easily in axenic culture under controlled conditions.

Of more immediate systematic interest, however, is the fact that not all ferns react to the antheridogens and different growth regimes in the same way (Table 1). Thus gametophytes of the first five families all produce antheridia in response to Antheridogen-A, while, with the exception of *Polypodium feei* (Polypodiaceae), the last four families show no response to the hormone. Antheridogen-B, in contrast, induces antheridia only in the Schizaeaceae. Similarly, the presence of gibberellic acid, GA_3, induces antheridial formation, but again only in the Schizaeaceae. However, Antheridogen-B and GA_3 induce different numbers of sperm in the antheridia of the same species.

In terms of the germination studies the spores of all ferns germinate in light. Most of the fern spores fail to germinate in the dark with the exception again of *Polypodium feei*, and *Polystichum munitum* (Aspidiaceae) and *Pteridium aquilinum* (Pteridaceae). However, if fern spores are grown in the dark but in the presence of Antheridogen-A, germination will occur in those species which normally show antheridial induction by Antheridogen-A. Similarly, spores of

the Schizaeaceae are the only ones which germinate in the dark in the presence of Antheridogen-B or GA_3. These results match those observed for antheridial induction in the families tested. Though incomplete, this work clearly shows the potential of systematic distinctions between families based on hormone physiology.

Using another approach, Glass and Bohm (1970), have tested for the phenol glucosylation reaction in ferns. It is known that angiosperms and gymnosperms possess the ability to absorb and glucosylate simple phenolic compounds when exogenously administered to the plants. Such abilities are apparently lacking in lower plant groups such as the algae, fungi, and bryophytes.

These workers selected the fern *Pityrogramma calomelanos* and *Psilotum nudum* for their feeding experiments. After administering large, exogenous quantities of the phenols, quinol and catechol, to these plants, large amounts of quinol and catechol glucoside were recovered from the plants. While only the two plant species were examined, the phenol glucosylation pathway does appear to be characteristic of vascular plants.

Since this synthetic ability appears in *Psilotum*, the authors conclude that this reaction pathway probably evolved quite early in plants, since *Psilotum* is considered to be an example of a primitive tracheophyte. However, if, as Bierhorst (1972) suggests, *Psilotum* is in fact a highly modified fern, then the occurrence of the phenol glucosylation reaction in *Psilotum* does not necessarily indicate an ancient evolutionary origin for this chemical reaction.

The next question is whether the reaction is present in other fern allies. Unfortunately, the authors did not test the lycopsids, but they do state that *Equisetum arvense* does possess this glucosylation pathway. This lack of data on the lycopods again leaves the phylogenist adrift.

Such a question is in fact raised in some recent work by Pokorny, Marčenko and Keglević (1970). These workers examined methionine metabolism in several species from each class of plants in the plant kingdom to determine if the metabolic pathways and/or metabolites derived from methionine metabolism were of systematic interest.

Radioactively labelled *L*- and *D*-methionine-methyl-^{14}C were administered under identical conditions in parallel experiments. The pathways of the two racemic forms of methionine during their metabolism to other amines and acids were examined using a combination of chromatography, electrophoresis, and radioactivity counting. The following points were discovered.

(1) The *L*-isomer of methionine is metabolized in the same way in all plants.

(2) The *D*-isomer, in contrast, can be metabolized in two different ways:

A. Algae, fungi, some lichens, and liverworts can convert the *D*-form into the *L*-form via deamination of the *D*-form, followed by racemization to the *L*-form, or, by direct *L*-specific reamination to the *L*-form.

B. Most higher plants (and some mosses) lack these pathways and the *D*-form is instead acylated to a malonyl conjugate of methionine with retention of the *D*-form.

(3) The fungi and lichen, though unable to form N-malonyl conjugates of methionine, can form N-acetyl conjugates of the *D*-form.

(4) The lycopods and some mosses are able to form both N-malonyl and N-acetyl conjugates and thus appear to be a transition series between vascular and non-vascular plants.

(5) While in vascular plants the major portion of the radioactive label appears in the N-malonyl-*D*-methionine conjugate, the non-vascular plants, in contrast, concentrate the radioactive label in non-conjugated acidic and neutral amine compounds. Again, some mosses and the lycopods seem to be transitional, accumulating the label in both conjugated and non-conjugated forms.

Such data certainly invite some speculation as to how close the relationship is between the mosses and the "primitive" tracheophytes. Unfortunately, *Psilotum* was not examined, and again the phylogenist is left in the dark as to its methionine synthesis and thus its probable phyletic position. The large scale phyletic implications of this type of physiological data are, however, quite clear.

Protein Studies

Systematic studies of ferns using proteins seem to be rather rare in the literature. However, a recent and very elegant study by Petersen and Fairbrothers (1970) on spore proteins in *Osmunda* deserves discussion. The problem involved whether or not *Osmunda claytoniana* is more closely related to *O. regalis* or to *O. cinnamomea*. Various systematists have favored one or the other of these relationships, while others favor neither and have raised all three taxa to distinct subgeneric or generic status. Using protein data the authors attempted to determine which of these possibilities is most probable. Comparisons of protein data were also made with *Matteuccia struthiopteris* and *Onoclea sensibilis* as well as *Dryopteris marginalis*.

The authors employed standard serological methods and more recently developed techniques, such as immunodiffusion, electrophoresis and immunoelectrophoresis, to ensure that their results were not due to the use of any one method. Further, they tested several different types of protein extraction methods to determine the full range of serological and electrophoretic reactions. Thus, they tested:

1) the crude protein extract from spores,
2) non-delipified extract in which lipid contaminants remained,
3) delipified extracts with all lipids removed, and
4) a protein extract partially purified by precipitation with ammonium sulfate.

Of these, the delipified protein extract gave the most clear-cut and consistent serological and electrophoretic results. Crude and non-delipified extracts were found to contain too many contaminants for good serological and electrophoretic resolution. Ammonium sulfate precipitation of protein extracts increased serological precipitation but decreased resolution of protein bands during electrophoresis. Further, there was some question as to whether protein precipitation for ammonium sulfate-treated extracts resulted from the increased concentration of just a few proteins or actually represented a true measure of all protein affinities between the taxa.

The results of this work using delipified protein extracts and both serological precipitates and electrophoretic band patterns, clearly show that *Osmunda clay-*

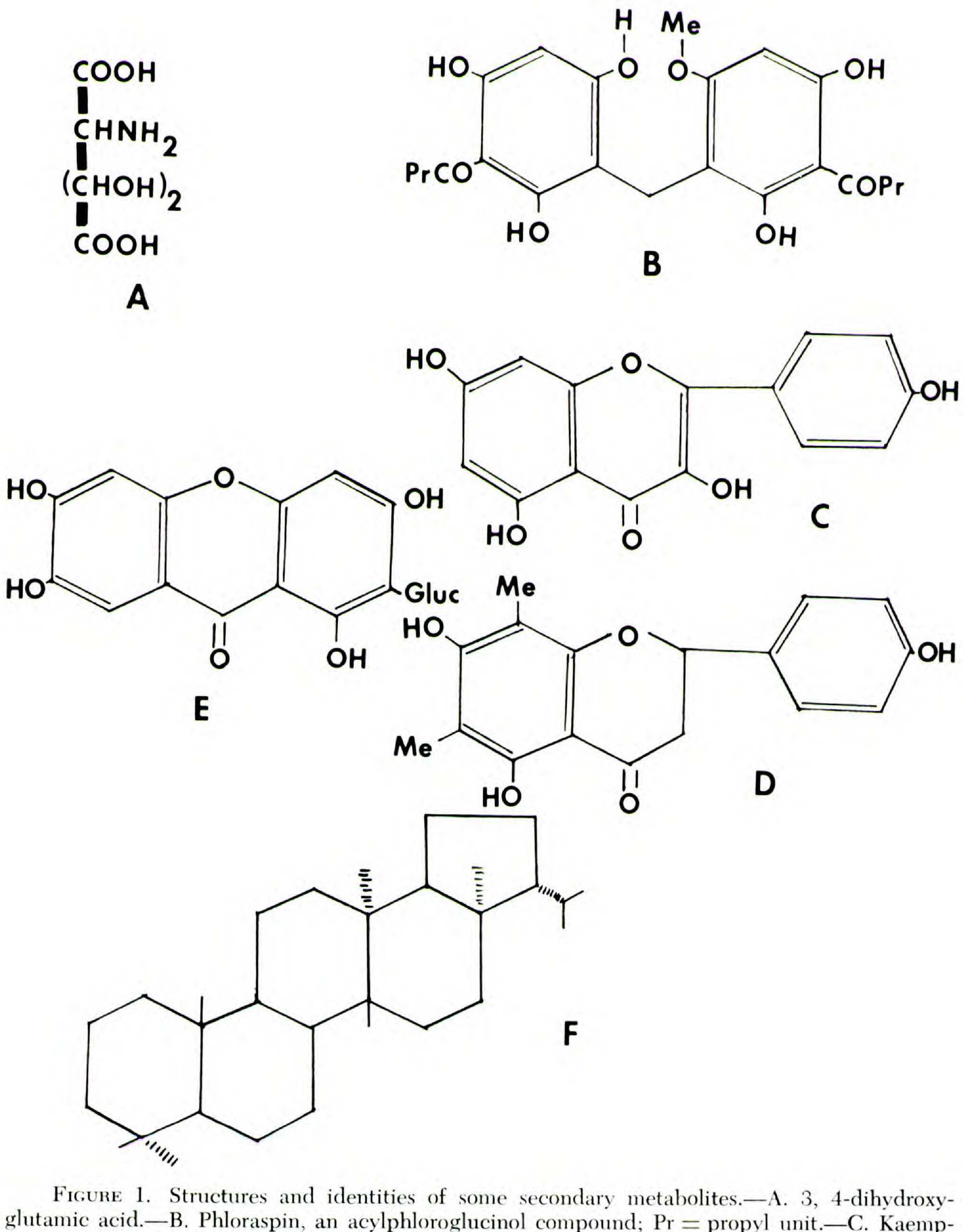

FIGURE 1. Structures and identities of some secondary metabolites.—A. 3, 4-dihydroxyglutamic acid.—B. Phloraspin, an acylphloroglucinol compound; Pr = propyl unit.—C. Kaempferol, a flavonol aglycone.—D. Farrerol, a C-dimethyl flavanone.—E. Mangiferin, a C-glucosyl xanthone.—F. Fernane, a triterpenoid; dotted constituents are in a plane opposite to that of the paper.

toniana is, in fact, more closely related to *O. cinnamomea*, and *O. regalis* is more closely related to *O. claytoniana* than to *O. cinnamomea*. The protein data further suggest that none of these taxa are worthy of subgeneric or generic rank.

Concerning generic comparisons, the authors found that *Osmunda* had greater protein affinities with *Matteuccia struthiopteris* and *Onoclea sensibilis* than with

Dryopteris. Dryopteris marginalis, in contrast, did show much protein affinity with *Matteuccia* and *Onoclea.*

While limited in scope, this work does set a detailed and rigorous testing model for further study of protein affinities in ferns.

Secondary Metabolites

While the physiological and protein studies show great potential in fern systematics, secondary metabolites probably represent the largest body of chemical data available for fern studies. These secondary metabolites (Bú Lock, 1961) accumulate in various tissues of plants. The range of variation in these secondary metabolites has recently been reviewed (Berti & Bottari, 1968), although the reader should consult Hegnauer (1962) for data published previous to 1961.

As an example, free amino acids such as 3, 4-dihydroxyglutamic acid (Fig. 1A) are often species-specific, in this case, to *Struthiopteris* [= *Matteuccia*] *filicastrum* (Berti & Bottari, 1968). Although a number of ferns have been screened for free amino acids, few phyletic conclusions above the generic level may be drawn from the data available (Panvisavas, Worthen & Bohm, 1968).

In the case of alkaloids, their presence in *Lycopodium* is well known (Hegnauer, 1962), although they appear to be absent in *Selaginella* and *Isoetes.* Similarly, a general survey for alkaloids in ferns indicates that they are absent from this group, too (Panvisavas, Worthen & Bohm, 1968). Thus, the systematic use of alkaloids would seem to be restricted to *Lycopodium.*

Triterpenoids have also been examined and identified in ferns, but the investigations have only begun since 1960, and it is too early to determine their systematic significance (Berti & Bottari, 1968). An example, fernane, is shown in Fig. 1F.

Another group of fern constituents, the acylphloroglucinol compounds (Fig. 1B), have been found only in the genus *Dryopteris*, where these compounds have been used extensively in chemosystematic and phylogenetic studies (Widén, 1971; Widén & Britton, 1971*a*, 1971*b*, 1971*c*).

One of the more interesting works in this series on *Dryopteris* is that on the *D. cristata* complex in North America (Widén & Britton, 1971*c*). The complex consists of a series of diploid species which have given rise to tetraploid and hexaploid derivatives. The evolution of these taxa from each other has been documented cytologically, and the acylphloroglucinol patterns in each taxon confirms its putative cytological origin. *Dryopteris cristata* itself is apparently derived from genomic contributions from *D. ludoviciana* of the *D. cristata* complex and another unknown diploid ancestor which has many characters in common with *D. spinulosa* of the *D. dilatata* complex. This hypothetical ancestor has been described and informally named "*D. semicristata*" or "*D. pseudo-spinulosa.*"

Examination of the chemistry confirms the similarities between *Dryopteris ludoviciana* and *D. cristata.* The chemistry also indicates that *D. spinulosa* of the *D. dilatata* complex is quite similar to *D. cristata* and that the hypothesized ancestor probably contributed genomes to both species. Correlation between

chemical and cytological data is very good; and since the identity of the compounds is known, the full value of the work is realized.

Flavonoids represent another large group of so-called secondary metabolites which have been used in chemosystematic studies, like those in *Dryopteris*. Unlike the acylphloroglucinol compounds, however, the flavonoids are not restricted to one genus but occur throughout the ferns as well as the fern allies (Hegnauer, 1962; Berti & Bottari, 1968). Like other fern constituents, though, large flavonoid surveys of the pteridophytes are lacking, and the systematic and phyletic value of flavonoids above the generic level remains undetermined as yet (Harborne, 1967).

Certainly, at the generic level and below, the flavonoids have proven to be most useful. The classic paper on the phenolics of *Asplenium* (Smith & Levin, 1963) still remains as an example of integration of cytological and chromatographic pattern data. Unfortunately, these authors did not identify the phenolics in *Asplenium*, and much chemical data of potential systematic interest is unavailable. Recently, however, Smith and Harborne (1971) have reexamined some of the compounds described in the earlier work. These first compounds were not flavonoids, but were mangiferin glycosides (Fig. 1E) which are xanthones.

Other early chromatographic studies also used only spot patterns (Scora & Wagner, 1964). However, the publication of several texts in recent years make the identification of flavonoids much easier; and thus there is little excuse for not including some identification of the compounds along with the pattern data (Mabry, Markham & Thomas, 1970; Jurd, 1962). Indeed, the importance of structural identification of flavonoids was emphasized in some recently completed work on the fern genera, *Gymnopteris* and *Hemionitis*, done by myself and Dr. John Mickel at the New York Botanical Garden.

These two genera have been maintained on the basis of their leaf morphology and marginal leaf venation. *Hemionitis* has palmate venation, and the fronds of most species are generally palmately lobed, while the architecture in *Gymnopteris* is generally pinnate. The two genera are also separated on the basis of whether the veins are free (*Gymnopteris*) or netted (*Hemionitis*). These characters, however, are not reliable, and distinctions break down upon close examination.

Examination of the flavonoids by two-dimensional paper chromatography shows that most of the species have their own specific profile (Table 2). However, it should also be noted that if these species are arranged in a manner based on their chemical similarity, regardless of their generic assignations, we find that two groups exist. It is immediately apparent that these chemical groupings cut across generic lines. The question then arises as to whether there actually is a structural homology between the compounds characterizing each group, and indeed there is.

Looking at Group II first, it is characterized by the presence of two or more of the compounds, 9–12 (Fig. 2A). Ultraviolet spectral analysis and comparison with authentic flavonoid samples, as well as published data (Mabry, Markham & Thomas, 1970; Jurd, 1962), show compounds 9 and 11 to be quercetin-3-

TABLE 2. Flavonoid distribution and spore types in *Gymnopteris* and *Hemionitis*.

Taxon	Flavonoid compounds 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Spore Type[a]
Group I																	
H. palmata	+	+			+	+	+	+									T
H. pinnatifida	+		+	+				+								+	T
H. levyi		+	+	+				+							+	+	T
G. rufa	+	+		+		+											T
Group II																	
H. elegans				+	+	+	+		+	+							C
H. arifolia	+			+				+									C
G. subcordata				+	+			+									C
G. bipinnata				+	+												C
G. vestita				+	+												C
G. tomentosa				+	+	+	+										C

[a] Spore types: T—tuberculate; C—crested.

rutinoside and 3-monoglucoside, respectively. Compounds 10 and 12 are kaempferol-3-rutinoside and 3-monoglucoside. All four of these pigments are typical flavonol glycosides (*cf.* Fig. 1C for kaempferol aglycone).

Group I, however, lacks these typical flavonols; and instead, the taxa in this group are characterized by possessing two or more of compounds 1–3 (*cf.* Fig. 2B), which, although not fully characterized, do appear to be different glycosides of a methylated flavonol aglycone (Mabry, Markham & Thomas, 1970). These data certainly suggest that the separation of these two genera on the aforementioned morphological grounds is unnatural.

Quite independently, Dr. Mickel had examined the spores of these same taxa using the scanning electron microscope. The micrographs showed two major spore types, those which were covered with spines or tubercles and those which have a series of crests or ridges on the surface.

When compared with the chemical data, all species of Group I with the methylated flavonol glycosides (compounds 1–3) have the tuberculate spores, while all species of Group II, with the more typical flavonols (compounds 9–12), have the crested spores (Table 2). The chemical and palynological data agree perfectly, and the arrangements cut across previous generic parameters. This has led to a recombination of the two genera and a redefinition of the genus *Hemionitis* (Mickel, 1973). In this case, both chemical and spore data have radically altered the disposition of these genera. Complete details of this work will be described in a subsequent paper.

CONCLUSIONS

The study of chemical phylogeny in the ferns, then, is most crucial, considering their phyletic position as the earliest vascular plants. Certainly, the methionine metabolism experiments indicate some metabolic similarities between the mosses and the lycopods, though much more proof is obviously needed to prove any direct relationship.

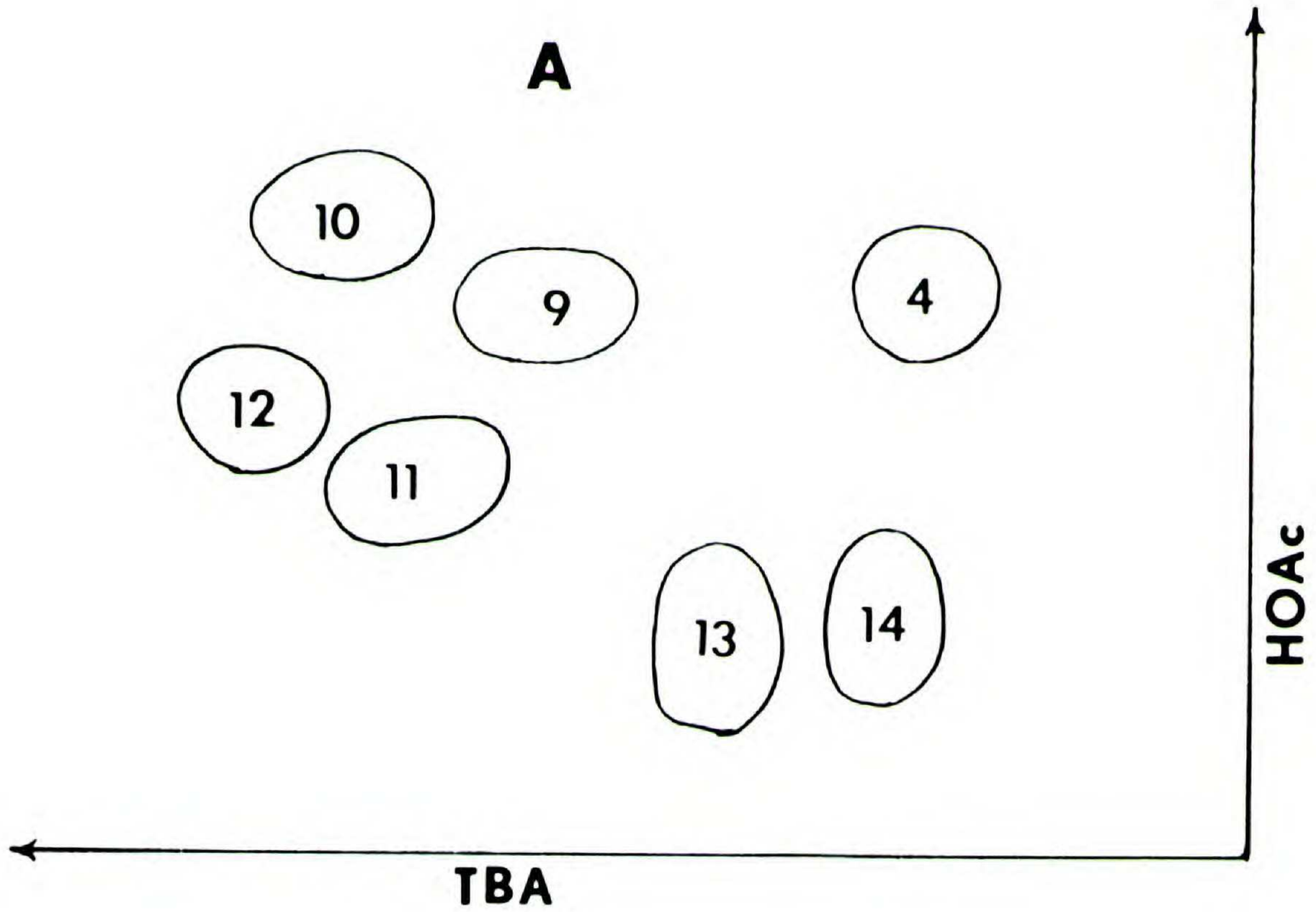

FIGURE 2. Chromatographic patterns of flavonoids in *Gymnopteris* and *Hemionitis*; TBA = *tert*-butanol: acetic acid: water (3:1:1 v/v); HOAc = acetic acid: water (15:85 v/v).—A. Composite chromatogram of flavonoids found in species in Group II (Table 2).—B. Composite chromatograms of flavonoids found in species in Group I (Table 2).

Of the three approaches discussed, each has its own strong points as well as its shortcomings. At the present time the use of secondary metabolites is perhaps the most profitable group of compounds to work with. Procedures for the identification of these compounds are available; and methods can be learned by the taxonomist, or, more profitably, by teamwork between the taxonomist and chemist. To date, however, surveys of this type are still incomplete; and only limited phyletic conclusions can be drawn. The physiological approach is probably more useful at the family level or higher, since it provides a direct comparison of primary metabolic processes. The techniques and equipment needed for such studies, though, are usually beyond the scope and training of most taxonomists.

Protein studies probably represent the truest approach to chemical phylogeny, since the proteins represent the primary translation products of the DNA code. Serology illustrates the first step in the systematic use of proteins. But again, the most valuable portion of this data, the amino acid sequence, still is not available for the ferns, though some limited information is available for angiosperms (Boulter, 1973). However, techniques and equipment are not sufficiently developed to allow for the rapid analysis of a large number of taxa (Turner, 1971).

Perhaps the ultimate use of molecular data to date may be found in the use of DNA annealing-hybridization comparisons between plants species (Voeller, 1971). DNA helices may be dissociated by heating and will reassociate upon cooling. Dissociated DNA strands can be mixed with similarly treated DNA from another taxon, the degree of relationship being determined by the percentage of hybrid renaturation. However, such techniques are quite complicated and, like previous biochemical approaches, require quite some expertise.

More important, perhaps, is the need for a scientific standard in which there is as careful and complete a selection as possible from all plant groups to be examined, regardless of the technique. This would do much to fill many of the empty holes in many phylogenetic schemes.

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